BEST AVAILABLE IMAGES

Defective images within this document are accurate representation of The original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

REMARKS

Claims 20 and 24-26 are pending in the subject application.

Rejection of Claim 20 under 35 U.S.C. §103(a)

Claim 20 stands rejected under 35 U.S.C. §103(a) as unpatentable over Olsson et al., 1983, J. Appl. Biochem. 5:437-445.

Applicants respectfully traverse this rejection.

The invention as set forth in claim 20 is:

"A method for diagnosing erythrocyte hemolysis in a subject who may have erythrocyte hemolysis comprising the steps of:

(a) obtaining a serum sample from said subject; and

(b) determining the level of erythrocyte adenylate kinase activity in said sample, the presence of at least about 20 U/L erythrocyte adenylate kinase activity in said sample being indicative of erythrocyte hemolysis in said subject."

Applicants maintain that Olsson does not teach or suggest each and every element of the claimed invention set forth in claim 20. In particular, Olsson does not teach determining the level of erythrocyte adenylate kinase activity in serum.

Olsson teaches measuring adenylate kinase activity by measuring the formation of ATP from ADP, using the firefly luciferase reaction (e.g. Olsson, bottom half of page 437). Thus, Olsson does teach measuring total adenylate kinase activity in a sample.

Olsson also teaches measuring adenylate kinase activity in the presence of DAPP (page 442, 2nd full paragraph). Olsson indicates that DAPP inhibits erythrocyte adenylate kinase activity and cites Szasz, G. et al. (Clin. Chem. 22: 1806-1811, 1976) in support of this statement. A copy of Szasz et al. (1976) is attached hereto. Olsson,

citing Szasz et al., further asserts that DAPP is a "specific inhibitor" of erythrocyte adenylate kinase. This last assertion is a clear misstatement of the teachings of Szasz et al. In contrast, Szasz et al. clearly state on page 1806, left column:

> "Diadenosine pentaphosphate inhibits erythrocyte and muscle adenylate kinase strongly ..., the liver isoenzyme less strongly. All three adenylate kinases may be present in a patient's serum ... . In acute hepatic damage, liver adenylate kinase activity in serum can be grossly increased."

Accordingly, Olsson teaches measuring either total adenylate kinase activity or the adenylate kinase activity remaining after the combined inhibition of erythrocyte, muscle and liver adenylate kinase activity (all three adenylate kinases can be present in serum). Olsson does not teach a way to determine the level of erythrocyte adenylate kinase activity in serum.

Furthermore, Szasz et al. provide a clear teaching away from relying on DAPP to distinguish erythrocyte adenylate kinase from total adenylate kinase in a serum sample.

The teachings of Olsson do not enable the skilled artisan to measure erythrocyte adenylate kinase activity in a serum sample and thus do not enable the skilled artisan to practice the claimed invention. In discussing obviousness, the U.S. Court of Appeals for the Federal Circuit has stated:

> The prior art must be enabling.[11] See *Motorola, Inc. v. Interdigital Tech. Corp.*, 121 F.3d 1461, 1471, 43 USPQ2d 1481, 1489 (Fed. Cir. 1997) ("In order to render a claimed apparatus or method obvious, the prior art must enable one skilled in the art to make and use the apparatus or method." (quoting *Beckman Instruments, Inc. v. LKB Produkter AB*, 892 F.2d 1547, 1551, 13 USPQ2d 1301, 1304 (Fed. Cir. 1989))). *Rockwell International Corp. v. United States*, 147 F.3d 1358, 1365, 47 USPQ2d 1027, 1032 (Fed. Cir. 1998).

Applicants maintain that Olsson is not enabling for the claimed invention, and therefore that the claimed invention is not rendered obvious in view of Olsson.

Accordingly, in view of the remarks made herein above, applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Rejection of Claims 24-26 under 35 U.S.C. §103(a)

Claims 24-26 stand rejected under 35 U.S.C. §103(a) as unpatentable over Olsson et al., 1983, J. Appl. Biochem. 5:437-445, as applied to claim 20, and further in view of Matsuura et al., 1989, J. Appl. Biochem. 264:10148-55.

Applicants respectfully traverse this rejection.

Applicants maintain that claim 20 is patentable over Olsson for the reasons set forth herein above. Claims 24-26 depend from, and further limit, claim 20. Accordingly, applicants maintain that claims 24-26 are patentable over the cited references.

Matsuura et al. teach the use of an antibody to adenylate kinase isozyme 1 (AK1) (Figure 10). Matsuura et al. teach that AK1 is present in the cytosol of skeletal muscle, brain and erythrocyte (see page 10148, right column, 1st paragraph). Accordingly, the antibody will detect not only erythrocyte adenylate kinase, but also muscle adenylate kinase, which as discussed herein above may be present in serum (Szasz et al., 1976), as well as any brain derived adenylate kinase that might be present in the serum.

Thus, neither Olsson nor Matsuura, either alone or in combination, teach or suggest each and every element of the claimed invention. In particular, neither teach determining the level of erythrocyte adenylate kinase in serum.

Accordingly, in view of the remarks made herein above, reconsideration and withdrawal of this ground of rejection are respectfully requested.

CONCLUSIONS

In view of the remarks made herein above, reconsideration and withdrawal of the rejections in the August 22, 2003 Final Office Action are respectfully requested. If there are any minor matters that would prevent allowance of the subject application, the Patent Office is requested to telephone the undersigned attorney.

No fee, other than the enclosed $860.00 fee for filing a Request for Continued Examination ($385.00) and Petition for a Three Month Extension of Time ($475.00) for a small entity, is deemed necessary with the filing of this response. However, if there are unanticipated fees required to maintain the pendency of this application, the PTO is authorized to withdraw those fees from Deposit Account 01-1785. Overcharges may also be credited to Deposit Account 01-1785.

Respectfully submitted,

AMSTER, ROTHSTEIN & EBENSTEIN LLP
Attorneys for Applicants
90 Park Avenue
New York, New York 10016
(212) 336-8000

Dated: New York, New York
February 13, 2004

By: 
Craig J. Arnold, Reg. No. 34,287
Alan D. Miller, Reg. No. 42,889

CLIN. CHEM. 22/11, 1806-1811 (1976)

Creatine Kinase in Serum: 2. Interference of Adenylate Kinase with the Assay

Gabor Szasz,[1] Willie Gerhardt,[2] Wolfgang Gruber,[3] and Erich Bernt[3]

Interference of adenylate kinase with Oliver's method [*Biochem. J.* 61, 116 (1955)] for creatine kinase is usually suppressed by including an adenylate kinase inhibitor, AMP. We studied the kinetics and compared the inhibition capacities of AMP and diadenosine pentaphosphate. Both are competitive inhibitors, AMP being markedly weaker, with a K_i of about 300 μmol/liter for adenylate kinase from erythrocyte, muscle, and liver. AMP also weakly inhibits creatine kinase. Diadenosine pentaphosphate inhibits erythrocyte and muscle adenylate kinase strongly (K_i about 0.03 μmol/liter), the liver isoenzyme less strongly (K_i about 3 μmol/liter), and has no effect on creatine kinase up to 100 μmol/liter. All three adenylate kinases may be present in a patient's serum, causing sample blanks to be high in a creatine kinase assay that lacks inhibitors. In acute hepatic damage, liver adenylate kinase activity in serum can be grossly increased. Use of sufficient diadenosine pentaphosphate alone for complete inhibition is relatively expensive. Consequently, we recommend a combination of both inhibitors. Diadenosine pentaphosphate, 10 μmol, combined with 5 mmol of AMP per liter inhibits adenylate kinase from erythrocytes and muscle by 97% and from liver by 95%.

There may be numerous interferences with the measurement of creatine kinase (ATP:creatine *N*-phosphotransferase; EC 2.7.3.2) activity (*3*) when the creatine phosphate → creatine reaction coupled with hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) is used (*1*).

For example, adenylate kinase (ATP:AMP phosphotransferase; EC 2.7.4.3), which catalyzes the reaction

$$2\,\mathrm{ADP} \overset{\text{adenylate kinase}}{\rightleftharpoons} \mathrm{ATP} + \mathrm{AMP}$$

can also act on the ADP, the substrate in the creatine kinase assay, to generate additional amounts of ATP and thus increase the apparent creatine kinase activity.

Adenylate kinase is widely distributed among the different organs, with decreasing activities in the sequence: skeletal muscle, brain, liver, heart, kidney, small intestine, spleen, and lung (*4*). In sera of healthy individuals the adenylate kinase activity ranges between 0–50 U/liter at 25 °C (*5*), but is markedly increased in hemolytic specimens.

Interference by adenylate kinase with the creatine kinase assay can be decreased by inhibiting adenylate kinase. Usually AMP has been used for this (*1*, *6–9*), but it not only inhibits adenylate kinase but also creatine kinase to some extent (*10*). Diadenosine pentaphosphate, a recently described potent inhibitor of adenylate kinase (*2*), selectively inhibits the enzyme of muscle and erythrocyte type at very low concentrations (10 μmol/liter); the liver and the kidney enzyme are less affected (*11*). Similar isoenzyme-specific inhibition of adenylate kinase has been also described for other compounds (*12*).

We compared the usefulness of AMP and diadenosine pentaphosphate as inhibitors of adenylate kinase in the creatine kinase assay, considering the selective inhibitory efficacy of diadenosine pentaphosphate and the incidence of the liver adenylate kinase isoenzyme in serum in various diseases.

Materials and Methods

Erythrocyte adenylate kinase was obtained from erythrocytes of healthy individuals. The erythrocytes were washed five times with physiological saline and then hemolyzed by adding equal volumes of doubly distilled water. The hemolysate was centrifuged at 15 000 × *g* for 2 min. In the clear supernates the hemoglobin concentration ranged between 110 and 140 g/liter of supernate.

Liver adenylate kinase was obtained from human biopsy material. A fresh sample of liver was weighed, homogenized in a chilled homogenizer (Heidolph Elektro KG, Kelheim, Germany; Typ RZR I) with 20 volumes of ice-cold physiological saline for 1 min, and centrifuged at 15 000 × *g* for 2 min. The adenylate kinase activity of the supernates ranged from 300 to 500 U/liter at 25 °C; the hemoglobin concentration was less than 20 mg per 100 ml of supernatant fluid.

Adenylate kinase from rabbit muscle and diadenosine pentaphosphate [P^1,P^5-di(adenosine-5′)pentaphos-

[1] Institute of Clinical Chemistry, University Medical School, Klinikstrasse 32b, 63 Giessen, Germany.
[2] Department of Clinical Chemistry, Lasarettet, 25187 Helsingborg, Sweden.
[3] Research Center, Boehringer Mannheim GmbH, 8132 Tutzing, Germany.

Presented in part at the 28th National Meeting of the AACC, Houston, Texas, August 1–6, 1976. *Clin. Chem.* 22, 1183 (1976). (Abstract.)
The first paper in this series is ref. *13* here.
Received June 28, 1976; accepted July 28, 1976.

phate) were from Boehringer Mannheim/Bio-Dynamics/bmc, Indianapolis, Ind. 46250.

Adenylate kinase activity was assayed at 25 °C in the German[1,3] and at 37 °C in the Scandinavian[2] laboratories, by the ADP → ATP reaction coupled with the hexokinase and glucose-6-phosphate dehydrogenase reactions (*1*). We used the reagents prescribed for a recently published optimized method for creatine kinase (*13*), except that we omitted creatine phosphate and AMP and started the reaction either with ADP or with serum. To establish the reference values, we measured the adenylate kinase activity under optimum conditions: ADP, 4 mmol/liter; magnesium acetate, 5 mmol/liter; glucose, 20 mmol/liter; $NADP^+$, 0.6 mmol/liter; hexokinase, 2500 U/liter; glucose-6-phosphate dehydrogenase, 1250 U/liter; triethanolamine, 250 mmol/liter; pH 7.6 at 25 °C. The adenylate kinase values obtained at the suboptimized conditions (*1*) with the creatine kinase assay reagent averaged 80% of the activity measured with the optimized method.

Creatine kinase activity was measured with an optimized modification (*13*) of Oliver's method (*1*). The activities of aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2), glutamate dehydrogenase (EC 1.4.1.2), and lactate dehydrogenase (EC 1.1.1.27) were assayed according to the Standard Methods of the German Society of Clinical Chemistry (*7*).

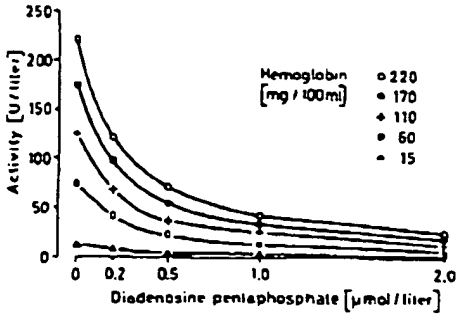

Fig. 1. Inhibition of adenylate kinase by diadenosine pentaphosphate
Human serum with admixed hemolysate

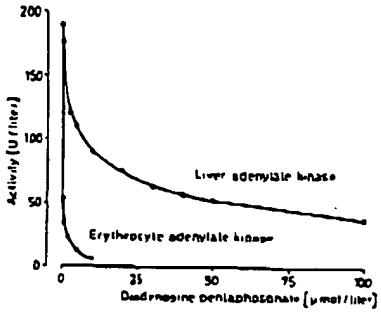

Fig. 2. Comparison of the inhibition of adenylate kinase from human liver and erythrocytes by diadenosine pentaphosphate

Results

Adenylate Kinase Activity in Serum in Health and Disease

To evaluate the potential adenylate kinase interference with the creatine kinase assay, we studied adenylate kinase activity in serum of healthy individuals and patients with muscle and liver damage.

Our reference group comprised 184 male and 204 female inpatients having normal serum creatine kinase, aspartate aminotransferase, and lactate dehydrogenase activities. In both groups the adenylate kinase values estimated at optimum conditions and at 25 °C showed nongaussian distributions. The 95% range of the reference values, calculated therefore by nonparametric statistics, was 8–53 U/liter for men and 8–47 U/liter for women.

To predict an increase in adenylate kinase activity by means of other enzyme activities is very difficult. We often observed normal adenylate kinase activities in sera of patients with various muscle lesions, even if the creatine kinase activities were highly increased, up to 1000 U/liter at 25 °C. Serial determinations in the course of acute myocardial infarctions yielded, during the first post-episode days, consistently normal adenylate kinase activities in half of the patients (peak creatine kinase values between 660 and 1690 U/liter at 25 °C); in the remaining cases we observed only slight and transitory elevations. The maximum values were between 71 and 83 U/liter; values for creatine kinase activity simultaneously were scattered between 520 and 730 U/liter. In hemolytic samples adenylate kinase activity may generally be assumed to be increased in proportion to the hemoglobin concentration.

Adenylate kinase from erythrocytes or muscle can be almost completely inhibited by 10 µmol of diadenosine pentaphosphate per liter (Figure 1), and consequently it does not interfere with creatine kinase assay when the assay mixture contains this compound in the appropriate concentration. However, it is important to know the incidence of liver adenylate kinase in serum, because this isoenzyme is inhibited only to 50% by 10 µmol of diadenosine pentaphosphate per liter (Figure 2).

We measured the adenylate kinase activity in sera of patients with various liver diseases. Table 1 presents a representative selection of our determinations. In acute hepatitis (patients 1–4) residual adenylate kinase activity, measured in the presence of 25 µmol of diadenosine pentaphosphate per liter, usually remained below 10 U/liter. We obtained similar results for five patients with chronic liver diseases (patients 5–9). In these patients (patients 1–9) we saw no correlation between the activity of adenylate kinase and of other enzymes, except for glutamate dehydrogenase.

The coincidence of the significant increase in glutamate dehydrogenase and adenylate kinase activity in patient 10 (Table 1) prompted us to further investigations. The predictive value of glutamate dehydrogenase activity could be indeed confirmed: highly increased glutamate dehydrogenase activities, above 100 U/liter, usually coincide with abnormal liver adenylate kinase values. In serum we found residual adenylate kinase activities between 10 and 70 U/liter, and in one patient, with acute congestion of the liver, the activity was 431

Table 1. Adenylate Kinase Activity in Sera of Patients with Pathological Results for Liver-Function Tests

Patient	Aspartate aminotransferase	Alanine aminotransferase	Glutamate dehydrogenase	Lactate dehydrogenase	Adenylate kinase[a]
	U/liter, at 25 °C				
Acute hepatitis					
1	998	660	34	390	8
2	576	976	26	323	7
3	404	597	51	536	11
4	666	984	22	434	8
Chronic liver disease					
5	125	259	30	252	5
6	60	105	36	595	5
7	49	38	6	178	3
8	25	25	6	662	6
9	44	27	8	616	7
Acute liver cell necrosis					
10	*561*	*411*	*336*	*860*	*30*
11	*3770*	*3460*	*309*	*5470*	*70*
12	*128*	*370*	*129*	*264*	*10*
13	*378*	*468*	*237*	*711*	*28*
14	*792*	*273*	*501*	*3900*	*65*
15	*828*	*1691*	*116*	*323*	*15*
16	*1545*	*657*	*352*	*6380*	*53*
17	*105*	*32*	*101*	—	*11*
18	*507*	*318*	*146*	*895*	*47*
19	*192*	*144*	*327*	*1580*	*18*
20	*3910*	*966*	*526*	*8950*	*431*

[a] Adenylate kinase activity was measured at optimum conditions in the presence of 25 μmol of diadenosine pentaphosphate per liter of assay mixture.

U/liter in the presence of 25 μmol of diadenosine pentaphosphate per liter.

Inhibition of Adenylate Kinase by AMP

To a human serum pool with low creatine kinase activity (34 U/liter at 25 °C) we added increasing amounts of hemolysate. We measured adenylate kinase activity with our standard reagent for creatine kinase assay (*13*), except that we omitted creatine phosphate and varied the AMP concentration. Thus the activities obtained correspond to the sample blank values of the creatine kinase assay. The adenylate kinase activity, without AMP in the assay mixture, proportionally increased with the portion of hemolysate in the sample (Figure 3). As an example, an amount of hemolysate corresponding to a hemoglobin concentration of 1 g/liter of sample, caused a sample blank activity of about 100 U/liter at 25 °C; at 37 °C the values were twice as high. This activity diminished with increasing AMP concentration. Even with as little as 1 mmol of AMP per liter there is only about half of the activity measured without AMP. This decreases to about 20, 12, and 7% at 3, 5, and 10 mmol of AMP per liter.

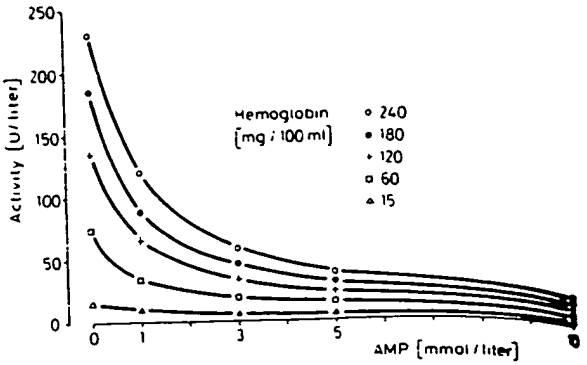

Fig. 3. Inhibition of adenylate kinase by AMP
Human serum with admixed hemolysate

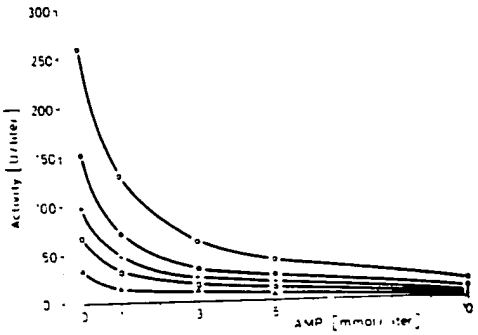

Fig. 4. Inhibition of adenylate kinase by AMP
Human serum enriched with rabbit muscle adenylate kinase

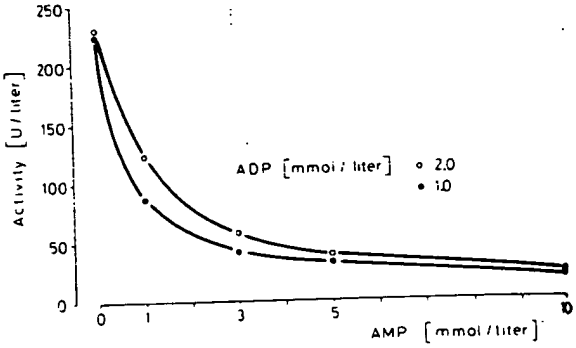

Fig. 5. Inhibition of adenylate kinase by AMP: dependence on ADP concentration
Human serum with admixed hemolysate

In another experiment, we added increasing amounts of purified adenylate kinase from rabbit muscle to the same serum pool, and measured adenylate kinase activity as specified above. The inhibition pattern was almost identical with that of the erythrocyte enzyme (Figure 4). The residual activities amounted to about 50, 25, 15, and 8% at 1, 3, 5, and 10 mmol of AMP per liter.

Inhibition of adenylate kinase by AMP depends on the ADP concentration (Figure 5). At 1 mmol of ADP per liter, residual activities measured in the presence of 1 mmol and 3 mmol AMP per liter, were only 38% and 18%, respectively, as compared to 51% and 22% at 2 mmol of ADP per liter. At 5 mmol and 10 mmol of AMP per liter, lesser differences were observed.

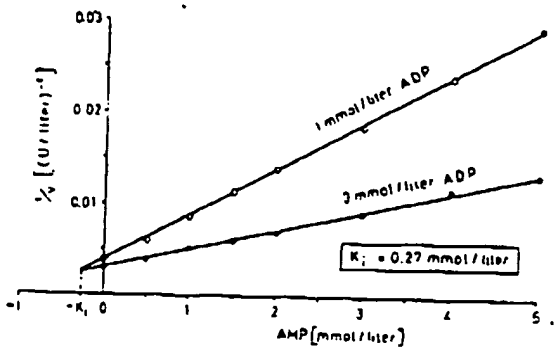

Fig. 6. Inhibition of adenylate kinase from human erythrocytes by AMP–Dixon plot

Adenylate kinase is competitively inhibited by AMP. The apparent K_i calculated from the Dixon plot was 0.27 mmol of AMP per liter for human erythrocyte adenylate kinase (Figure 6) and 0.38 mmol AMP per liter for human liver adenylate kinase (not illustrated) at 25 °C assay temperature. These values were essentially confirmed on using specimens from other patients.

Inhibition of Creatine Kinase by AMP

Creatine kinase activity was measured in nonhemolyzed sera in the presence of 2 mmol of ADP per liter and at various AMP concentrations. At each AMP concentration the individual sample blanks were also measured in the absence of creatine phosphate, starting the reaction with ADP. "True" creatine kinase activities were calculated by subtraction of the individual sample blanks. The inhibition of the "true" creatine kinase activity averaged 3, 5, 10, 17, and 30% at 3, 5, 10, 20, and 40 mmol AMP per liter, respectively.

Clearly, the inhibition depended on the AMP:ADP molar ratio. At a ratio of 5, e.g., it amounted to about 10%, independent of the actual molar concentration of the compounds (5:1 or 10:2). In accordance with this, AMP showed a competitive inhibition pattern with ADP in the Dixon plot (not illustrated), the K_i for AMP being about 10 mmol/liter at 25 °C. On the other hand, with creatine phosphate as the variable substrate, AMP gives an uncompetitive inhibition pattern. We have not invested more work in the study of the nature of creatine kinase inhibition by AMP because of its minor practical significance for creatine kinase assay.

Inhibition of Adenylate Kinase by Diadenosine Pentaphosphate

Human erythrocyte adenylate kinase was strongly inhibited by diadenosine pentaphosphate (Figure 1). As in the inhibition experiments with AMP, we enriched a human serum pool with increasing amounts of hemolysate. Inhibition averaged 45, 90, 94, and 97% at 0.2, 2, 5, and 10 μmol of diadenosine pentaphosphate per liter. The pattern of inhibition of rabbit muscle adenylate kinase by diadenosine pentaphosphate was almost the same.

In contrast, adenylate kinase from human liver was relative weakly inhibited by diadenosine pentaphosphate. The extent of inhibition amounted only to about 50%, 70%, and 80% at 10, 50, and 100 μmol of diadenosine pentaphosphate per liter (Figure 2). For complete inhibition of liver adenylate kinase 500–1000 μmol of diadenosine pentaphosphate per liter was necessary.

Diadenosine pentaphosphate competitively inhibited adenylate kinase. The apparent K_i values, calculated from Dixon plots like that in Figure 6, were 28 nmol of diadenosine pentaphosphate per liter for adenylate kinase from human erythrocytes but 2.9 μmol of diadenosine pentaphosphate per liter for human liver adenylate kinase.

Table 2. Effect of Inhibitors on the Sample Blank Value of the Creatine Kinase Assay

Patients	Without inhibitor	AMP (5 mmol/liter)	AMP (5 mmol/liter) and diadenosine pentaphosphate (10 μmol/liter)
	U/liter, at 25 °C		
Controls (n = 12)	12 (6–27)	6 (4–15)	5 (2–8)
Muscle damage (n = 12)	18 (8–34)	6 (4–11)	4 (4–8)
Liver disease (n = 12)	18 (11–30)	9 (4–15)	8 (4–15)
Hemolytic sera (n = 12)	61 (19–101)	15 (8–19)	8 (3–11)

Means and ranges (in parentheses) of sample blank (without creatine phosphate).

Inhibition of Creatine Kinase by Diadenosine Pentaphosphate

Creatine kinase activity was measured in eight nonhemolyzed sera (75–260 U/liter at 25 °C) with our standard reagent (*13*) containing diadenosine pentaphosphate in various concentrations (0, 10, 100, 500, and 1000 μmol/liter). Up to a concentration of 100 μmol of diadenosine pentaphosphate per liter we observed no inhibition of the "true" creatine kinase activity at all. Inhibition ranged from 0–3%, and 1–5% at 500 and 1000 μmol of diadenosine pentaphosphate per liter of assay mixture, respectively. As in the inhibition study with AMP, the "true" creatine kinase activities were calculated by subtraction of the individual sample blanks.

Effect of Inhibitors on the Sample Blank of the Creatine Kinase Assay

The sample blanks were measured without and with inhibitors at 25 °C and 37 °C, omitting creatine phosphate from the standard creatine kinase reagent (*13*). Five millimoles of AMP per 100 ml of assay mixture decreased sample blank rates by at least half in nonhemolyzed sera from patients, even those with normal enzyme profiles (Table 2). Additional diadenosine pentaphosphate (10 μmol/liter) did not influence the mean sample blank. In one serum the sample blank

decreased in the sequence: 27 U/liter without inhibitor, 15 U/liter with AMP, and 8 U/liter in the presence of both inhibitors. The presence of muscle adenylate kinase in this sample seemed unlikely because the creatine kinase activity was normal (23 U/liter at 25 °C). We assume that erythrocyte adenylate kinase was present, although this group comprised only sera without visible hemolysis. This phenomenon was sporadically but repeatedly observed.

At 37 °C, sample blank values were about twice as high as at 25 °C. Even in the absence of visible hemolysis and without any inhibitors, values as high as 40–60 U/liter were observed. These blank values, found in 10 sera, were decreased by the presence of 5 mmol of AMP per liter to 9–14 U/liter. Additional diadenosine pentaphosphate (10 μmol/liter) further decreased the blank rates to 4–7 U/liter.

The sample blanks could be significantly decreased also in sera of patients with muscle damage (creatine kinase: 110–1630 U/liter at 25 °C) and liver diseases (pathological liver enzyme profile) by the use of inhibitors. If both inhibitors were included, there was an additional decrease in the sample blank of sera containing muscle adenylate kinase. As expected, no effect at all was observed by additional diadenosine pentaphosphate on the sample blank of sera with liver adenylate kinase (Table 2).

The usefulness of the inhibitor mixture was further elucidated for sera with even faint hemolysis. Addition of 10 μmol of diadenosine pentaphosphate per liter decreased the sample blank by almost half as compared with the sample blank measured only in the presence of 5 mmol of AMP per liter (Table 2).

In another experiment at 25 °C, increasing amounts of hemolysate were added to a serum with low creatine kinase activity. The effect of hemolysis on the sample blank and on the apparent creatine kinase activity is demonstrated in Figure 7. Both the sample blank and the apparent creatine kinase activity simultaneously increased with the ratio of hemolysate. AMP greatly diminished interference from erythrocyte adenylate kinase, but the residual activity (about 15%) could lead

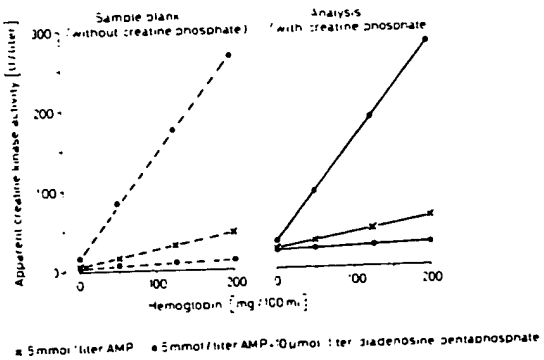

Fig. 7. Effect of hemolysis on the sample blank and on the apparent creatine kinase activity in the absence of inhibitors (O), in the presence of 5 mmol AMP per liter (x) and additional 10 μmol diadenosine pentaphosphate per liter (●)
Human serum with admixed hemolysate

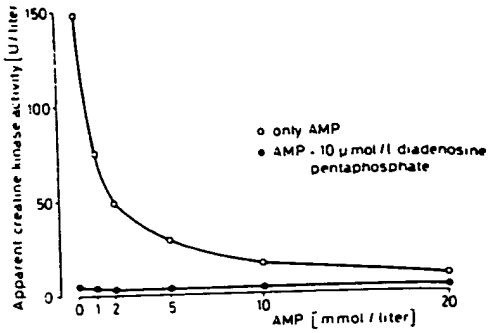

Fig. 8. Reduction of the sample blank of a hemolyzed serum by AMP as single inhibitor (O) and by AMP with additional diadenosine pentaphosphate (10 μmol/liter) (●)

to a clinically significant falsification of the creatine kinase value. The apparent creatine kinase activity increased from 23 U/liter without admixed hemolysate to 34, 48, and 62 U/liter at hemolysate admixtures corresponding to 500, 1250, and 2000 mg of hemoglobin per liter of serum, but to only 24, 26, and 27 U/liter when diadenosine pentaphosphate was also incorporated in the assay mixture.

At 37 °C we obtained similar results (Figure 8). In hemolyzed serum the initial sample blank in the absence of inhibitors was decreased by 97% (from 148 U/liter to 4 U/liter) by a combination of 5 mmol of AMP and 10 μmol diadenosine pentaphosphate per liter, as compared to a decrease of only 80% (from 148 U/liter to 29 U/liter) by 5 mmol AMP per liter alone. Even at 20 mmol of AMP per liter (i.e., an AMP:ADP molar ratio of 10:1) the sample blank was only reduced by 93%.

Discussion

Our experiments confirm the significant effect of adenylate kinase on the creatine kinase activity measurement (2) and emphasize the need to suppress it. Interference from adenylate kinase can be blanked out by an individual sample blank in which creatine phosphate is omitted from the reagent (*10*), but this procedure not only doubles the workload but also diminishes the precision of the assay because the imprecision of both analysis and blank will influence the final result. That is why most authors prefer to inhibit adenylate kinase with AMP (*1, 6–9*). Oliver (*1*) found that adenylate kinase is completely inhibited when the AMP concentration is 10-fold that of ADP. This AMP:ADP ratio was also considered to be satisfactory by other investigators (*6–8, 15*). Inhibition of creatine kinase by AMP was generally assumed to be negligible (*1, 6, 15*); only Hess et al. (*10*) described a marked inhibition, at an AMP:ADP ratio of 10:1.

In our study, the sample blank of hemolyzed sera containing 1 g of hemoglobin per liter had an apparent creatine kinase activity of about 100 U/liter at 25 °C. This value could be significantly decreased by incorporating AMP in the reagent. AMP competitively inhibits adenylate kinase. The association constants for the binding of AMP to adenylate kinase ranged around 0.3 mmol/liter and were not significantly different for

the erythrocyte and liver enzyme. Adenylate kinase was 95% inhibited at an AMP:ADP ratio of 10:1, but creatine kinase was simultaneously inhibited by 15–20%. At the AMP:ADP ratio of 5:2 we recommend (*13*) the inhibition of creatine kinase is still about 5%, but interference by adenylate kinase is decreased to only 10–15%.

A very promising alternative to AMP seemed to us to be diadenosine pentaphosphate (*2*) as inhibitor of adenylate kinase in the creatine kinase assay. We confirmed the high inhibitory efficacy of this compound on adenylate kinase from erythrocytes and muscle. Adenylate kinase from human erythrocytes and rabbit muscle was almost completely inhibited already at 10 μmol of diadenosine pentaphosphate per liter, whereas creatine kinase was not affected at all by this compound up to 100 μmol per liter. The inhibitory power on human liver adenylate kinase was less marked. Even at 100 μmol of diadenosine pentaphosphate per liter we observed a residual activity of about 20%. For complete inhibition, concentrations of 500–1000 μmol/liter are necessary. Accordingly the K_i value was 100-fold higher for the liver enzyme than for the erythrocyte enzyme.

These results raised the question about the prevalence and extent of liver adenylate kinase activity in serum—i.e., how concerned one should be about interference with the creatine kinase determination. Data on adenylate kinase in liver diseases are scarce (*5*); we could find no information on the liver isoenzyme in serum. In serum from cases of acute and chronic liver diseases, we saw residual activities of as much as 10 U/liter when 25 μmol of diadenosine pentaphosphate per liter was incorporated in the reagent. Moreover, acute liver cell damage, indicated by marked increase in the activity of glutamate dehydrogenase, an enzyme exclusively localized in the mitochondria (*16*), coincided with a significant increase in liver adenylate kinase. We measured residual activities as great as 70 U/liter—and in one case with acute congestion of the liver even 431 U/liter—with a reagent containing 25 μmol of diadenosine pentaphosphate per liter.

To summarize: Diadenosine pentaphosphate appears to be an ideal inhibitor for the muscle and erythrocyte type of isoenzymes of adenylate kinase. Unfortunately, the liver enzyme is less suppressed, and relative high activities may be expected in cases with acute liver cell damage. The use of diadenosine pentaphosphate alone in a sufficiently high concentration was precluded because this compound is very expensive. On the other hand, with AMP alone and an AMP:ADP molar ratio of 5:2, adenylate kinase will be inhibited only to 80–85% independently of the isoenzyme composition. A further increase in AMP concentration would increase the inhibition of creatine kinase. The combination of both inhibitors, however, appears to be a good choice.

Note added in proof: After this manuscript had been submitted Rosano et al. [*Clin. Chem.* 22, 1078 (1976)] recommended sodium fluoride as inhibitor of adenylate kinase in the creatine kinase assay. In our experiments, 25 mmol of fluoride per liter assay mixture inhibited adenylate kinase from human erythrocytes to 92% and from human liver to 83% after a lag phase of 4 min at 30 °C as recommended by Rosano et al. The rate of inhibition increased until a lag phase of 6–8 min and amounted to 95% or 88%, respectively. We simultaneously found a mean inhibition of creatine kinase of 6% in 18 human sera (creatine kinase activity: 60–320 U/liter at 30 °C) by 25 mmol of fluoride per liter.

Another attempt to omit AMP completely failed also. Diadenosine tetraphosphate and diadenosine hexaphosphate (P-L Biochemicals Inc., Milwaukee, Wis. 53205) proved to be even weaker inhibitors of both erythrocyte and liver adenylate kinase than was diadenosine pentaphosphate.

We gratefully recognize the competent technical assistance of Miss Elfriede Kinne and Mrs. Birgit Hedenäs, and the help of Mr. Harald Glaucke, Miss Eva-Beate Nittel, and Mr. Lucky Hettler with the manuscript. For generously providing human liver biopsy material we are indebted to Prof. Dr. B. Leinweber. This study was supported by research grants of the Deutsche Forschungsgemeinschaft and the Segerfalska Stiftelsen.

References

1. Oliver, I. T., A spectrophotometric method for the determination of creatine phosphokinase and myokinase. *Biochem. J.* 61, 116 (1955).

2. Lienhard, G. E., and Secemaki, I. I., P^1,P^5-Di(adenosine-5')pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase. *J. Biol. Chem.* 248, 1121 (1973).

3. Szasz, G., Laboratory measurement of creatine kinase activity. In *Proceedings of the Second International Symposium on Clinical Enzymology*, N. W. Tietz, A. Weinstock, and D. O. Rodgerson, Eds. 1975. In press (Amer. Assoc. Clin. Chem.).

4. Lahmann, F. G., Schneider, K. W., and Menge, H., Die enzymatische Diagnostik des Herzinfarktes. II Mitteilung: Die Bestimmung von organspezifischen Enzymen: Kreatinphosphokinase und Myokinase. *Enzymol. Biol. Clin.* 6, 36 (1966).

5. Madritsch, K., Die Myokinase in der Diagnostik des Herzinfarktes. *Schweiz. Med. Wochenschr.* 98, 646 (1968).

6. Rosalki, S. B., An improved procedure for serum creatine phosphokinase determination. *J. Lab. Clin. Med.* 69, 696 (1967).

7. Recommendations of the German Society of Clinical Chemistry. Standardization of methods for the estimation of enzyme activities in biological fluids. *Z. Klin. Chem. Klin. Biochem.* 8, 659 (1970).

8. Swanson, J. R., and Wilkinson, J. H., Measurement of creatine kinase activity in serum. *Stand. Methods Clin. Chem.* 7, 33 (1972).

9. Warren, W. A., Activation of serum creatine kinase by dithiothreitol. *Clin. Chem.* 18, 473 (1972).

10. Hess, J. W., Murdock, K. J., and Natho, G. J. W., Creatine kinase—A spectrophotometric method with improved sensitivity. *Am. J. Clin. Pathol.* 50, 89 (1968).

11. Sachsenheimer, W., Goody, R. S., and Schirmer, R. H., Elimination und Exkretion von Adenylat-Kinasen nach Zellschädigung. *Klin. Wochenschr.* 53, 617 (1975).

12. Russel, P. J., Jr., Horenstein, J. M., Goins, L., et al., Adenylate kinase in human tissues. I. Organ specificity of adenylate kinase isoenzymes. *J. Biol. Chem.* 249, 1874 (1974).

13. Szasz, G., Gruber, W., and Bernt, E., Creatine kinase in serum: 1. Determination of optimum reaction conditions. *Clin. Chem.* 22, 650 (1976).

14. Hess, J. W., MacDonald, R. P., Natho, G. J. W., and Murdock, K. J., Serum creatine phosphokinase: Evaluation of a commercial spectrophotometric method. *Clin. Chem.* 13, 994 (1967).

15. Wilkinson, J. H., and Steciw, B., Evaluation of a new procedure for measuring serum creatine kinase activity. *Clin. Chem.* 16, 370 (1970).

16. Schmidt, E., and Schmidt, F. W., Methode und Wert der Bestimmung der Glutaminsäure-Dehydrogenase-Aktivität im Serum. *Klin. Wochenschr.* 40, 962 (1962).